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THE EFFECT OF MECHANICAL VIBRATION ON ALVEOLAR BONE FOLLOWING EXPERIMENTAL PERIODONTITIS – A TIME COURSE STUDY

by

Joshua M. Murphy, D.M.D.

A Thesis submitted to the Faculty of the Graduate School,
Marquette University,
in Partial Fulfillment of the Requirements for
the Degree of Master of Science

Milwaukee, Wisconsin

August 2020

ABSTRACT
THE EFFECT OF MECHANICAL VIBRATION ON ALVEOLAR BONE FOLLOWING EXPERIMENTAL PERIODONTITIS – A TIME COURSE STUDY

Joshua M. Murphy, D.M.D.

Marquette University, 2020

Introduction: High-frequency vibration with low magnitude acceleration has varying effects on alveolar bone. The objectives of this study were to establish a murine model for periodontitis and to explore the best time window of this model to investigate the effects of high frequency, low magnitude mechanical vibration on alveolar bone following ligature-induced experimental periodontitis.

Materials and Methods: Ninety-five 11-week-old inbred strain C57BL/67 male mice were randomly assigned into four groups: 1) healthy control (n = 9); 2) healthy + mechanical vibration (n = 8); 3) experimental periodontitis + no treatment (n=7); and 4) experimental periodontitis + vibration (n = 9). All mice in the disease groups had ligature-induced experimental periodontitis induced for 8 days to generate localized alveolar bone loss. In mechanical vibration treatment groups, the mice received high frequency mechanical vibration (60 Hz, 0.3 g) for 5 min/day on the maxillary right 1st molar for consecutive 7 and 21 days, respectively to determine the effects on alveolar bone following experimental periodontitis. Micro computed tomography (micro-CT) was used to quantify new bone formation through bone volume fraction (BVF), tissue mineral density (TMD), and alveolar bone heights post treatment with or without mechanical vibration. Analysis of variance (ANOVA) was performed with Bonferroni post hoc tests to measure statistically significant differences between groups for volumetric and linear bone levels. P value less than 0.05 was considered significant.

Results: Ligature-induced experimental periodontitis resulted in significant reductions in BVF, TMD and alveolar bone height compared to healthy controls. Treatment with mechanical vibration for 7 and 21 days led to a non-significant, local anabolic effect; however, decreases in BVF and TMD of alveolar bone were seen in areas adjacent to the site of application of mechanical vibration.

Conclusion: Healing in ligature-induced experimental periodontitis is in progress at 7 days and completed by 21 days. Mechanical vibration (60 Hz, 0.3 g, 5 min/day) modestly increases bone volume and density of the tooth vibrated directly, indicating a potential clinical application for improving bone quantity and quality following periodontitis.

ACKNOWLEDGEMENTS

Joshua M. Murphy, D.M.D.

I would first like to express my gratitude to Dr. Dawei Liu for his belief and support not just in my research, but in my career as an orthodontist. I would also like to extend my respect and thanks to Dr. Andrei Taut who did the animal handling and established the needed protocols. This thesis would not have been possible without his efforts, the support and advice of Dr. Julie Marchesan at the University of North Carolina – Chapel Hill, or the statistical expertise of Dr. Maharaj Singh. Furthermore, I would like to sincerely thank the rest of my committee, Dr. Bhoomika Ahuja, Dr. Ghada Nimeri. They have both been involved and influential in my time at Marquette and my progression towards my graduation and future career.

No one has been more important to me in the pursuit of my degree and this thesis than my family. I am grateful to my siblings and parents – whether by blood, unanimous agreement, or marriage – for their support and interest in my pursuits. Most importantly, I want to thank my patient and supportive wife Hailee and my children Benjamin, Miles, and Oliver. They have never stopped supporting me through the many long days, the late nights, and even months spent apart. Nothing means more to me than their love and support.

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CHAPTER I – INTRODUCTION

There are increasing numbers of people and teeth with periodontitis (Eke, Borgnakke, & Genco, 2020). Tooth loss is a significant consequence of periodontal disease/alveolar bone loss that can significantly reduce Oral Health-Related Quality of Life in affected patients (Gerritsen, Allen, Witter, Bronkhorst, & Creugers, 2010). Once periodontal disease is under control, regeneration of alveolar bone can be performed using surgical and pharmaceutical treatments; however, such treatments are often invasive, costly, and are limited to small regions of bone loss. Though the prevention of periodontal disease is being addressed, there remains a need for an effective, non-invasive, and safe treatment for alveolar bone loss to address this public health concern and help maintain the oral health and quality of life of those affected by periodontitis.

Periodontal disease is estimated to affect 42.2% of the adult population of the United States with 7.8% of people experiencing severe periodontitis (Eke et al., 2020), and is a chronic inflammatory disease caused by bacterial toxins. This inflammation affects gum tissue and alveolar bone and leads to tooth loosening and even tooth loss (American Academy of Periodontology, 2019). Furthermore, periodontal disease has a negative impact on systemic health and has been linked to serious conditions such as cardiovascular disease, strokes, and even Alzheimer's disease (Miricescu et al., 2019; Singhrao & Olsen, 2019).

Successful management of periodontal disease can reduce the risk of systemic complications, but alveolar bone is difficult to regenerate. It can be accomplished using surgical and pharmaceutical treatments; however, such treatments are often invasive,

costly, and are limited to small regions of bone loss. Even if patients are willing to undergo more invasive treatments, the clinical success for periodontal regeneration still remains limited in many cases (Giannobile, Lang, Lindhe, Sanz, & Berglundh, 2015). Mechanical vibration has been shown to increase alveolar bone density in mice (Yadav et al., 2015). However, no knowledge is available on the effects of mechanical vibration on the regeneration of alveolar bone in periodontitis. Therefore, the aims of this study are 1) to establish a mouse periodontitis model and 2) to investigate the effects of high frequency, low magnitude mechanical vibration on alveolar bone following ligature-induced experimental periodontitis.

CHAPTER II –LITERATURE REVIEW

PERIODONTAL BONE LOSS

Periodontal disease consists of a complicated interaction between bacterial pathogens and host responses that leads to damage and loss of tooth-supporting hard and soft oral tissues (Hasan & Palmer, 2014). It is widely established the periodontal disease is initiated by accumulation of bacterial pathogens as dental plaque (Graves, D. T., Li, & Cochran, 2011). A variety of different bacteria are involved throughout the development of periodontal disease. Gram-Positive organisms begin the colonization, but, over time, there is a shift in microflora from Gram-positive to Gram-negative organisms (Hasan & Palmer, 2014).

The bacterial flora that cause periodontal disease are thought to cause bone loss and other destruction through two different mechanisms: direct and indirect action. Hasan and Palmer describe early lesion as having more direct action which is where the microorganisms or their products affect host tissues. Some examples of this mechanism are: damage to crevicular epithelium, leukocyte impairment by leukotoxin, impairment of polymorphonuclear leukocyte (PMN) function, dysregulation of cytokine networks, degradation of immunoglobulins and fibrin, increase in mucosal permeability and disaggregation of proteoglycans, breakdown of periodontal tissues, activation of complement and bone resorption by endotoxin (LPS), and bone resorption stimulated by lipoteichoic acid (Hasan & Palmer, 2014). This initial damage to host tissues and disruption of host processes allows colonization to continue and causes progression of the disease.

As the periodontal lesions progress, the mechanism of host-tissue destruction shifts from direct to indirect. Indirect damage is classified as damage that comes as a result of microflora initiating host inflammatory responses causing damage tissue (Hasan & Palmer, 2014). Graves et al. reviewed various classes of molecules that can stimulate bone resorption through osteoclastogenesis including such lipid-based mediators as prostaglandins, cytokines, and chemokines. One such cytokine is Interleukin-1 which significantly contributes to pathologic bone loss through upregulation of receptor activator for nuclear factor kappa-B (RANK) ligand which is known to stimulate osteoclastogenesis and bone resorption (Graves, D. T. et al., 2011). Other host responses are also implicated in the hard tissue destruction caused by periodontal disease. Some examples given by Hasan and Palmer are: activation of B cells which prevents adaptive immune responses from targeting destructive antigens, release of cytokines caused by activating T-cells, and recruitment of PMNs that release destructive enzymes (matrix metalloproteinases) (Hasan & Palmer, 2014). These various mechanisms of indirect damage explain the variance seen in the destructive potential of periodontal disease due to various risk factors such as inherited host factors, lifestyle, age, systemic disease, genetics, stress, and trauma (Giannobile et al., 2015).

Whether the soft and hard tissue destruction is caused by direct or indirect mechanisms, Graves et al. concluded that the central issue is not so much the qualitative nature of the present inflammation, but the proximity of the inflammation to the bone. The normal coupling of bone resorption and bone formation is disrupted through the indirect mechanism of the pathogens likely as a result of pro-inflammatory cytokines.

These cytokines reduce bone formation and increase resorption causing bone loss and reduced tooth support (Graves, D. T. et al., 2011).

RECONSTRUCTIVE PERIODONTAL THERAPIES

Treatment for periodontal disease is performed using various procedures and techniques. Initial therapy is intended to arrest the progression of the disease through removal of sub-gingival and supra-gingival plaque using scaling and root planning (Giannobile et al., 2015). Removal of plaque in conjunction with appropriate maintenance by the patient can stop progression of the disease, but surgery is often required for pocket reduction via gingivectomy or flap surgery (Giannobile et al., 2015). Without pocket reduction, many patients will be unable to maintain an appropriate level of oral hygiene to prevent future disease (American Academy of Periodontology, 2019). Once periodontal disease has been controlled, there is minimal recovery of hard and soft tissue damage without further intervention through regenerative procedures. The American Academy of Periodontology defines regeneration as, “Reproduction or reconstitution of a lost or injured part in a manner similar or identical to its original form” (American Academy of Periodontology, 2019). Regenerative procedures are an ideal treatment following periodontal disease as they generate recovery of tissues that the body will not regenerate on its own: such as cementum, bone, and the periodontal ligament, including Sharpey’s fibers (Floyd, Ide, & Palmer, 2014). This tissue regeneration is so important because it leads to the overall improvement of function, esthetics, and health of the teeth and surrounding periodontium. Though the regeneration of hard and soft tissues are thoroughly connected, the regeneration of hard tissue is the more relevant of the two to the purposes of this study and will thus be explored in the most detail.

Bone loss in the oral cavity is typically treated through bone grafting, but the techniques and types of grafts vary. The general categories of grafts are differentiated by the source of the graft material. Autogenous grafts are harvested from the same patient in a different location, allogenic grafts come from the same species but a different donor, xenogenic grafts are from different species, and alloplastic material grafts are synthetic or inorganic materials (Giannobile et al., 2015). The previous understanding of regeneration through hard tissue grafts was that cells derived from the bone were able to form cementum and insert new collagen fibers on the root surface, but this perception has been replaced with the more current understanding that the periodontal ligament is the prerequisite for the formation of new attachment (Giannobile et al., 2015). Though widely used, and generally considered to be effective, bone grafting as a method of periodontal regeneration draws most of its scientific support from case reports, and has limitations such as decreased success with fewer walls of existing bone, lengthy surgeries, and initial resorption of exposed bone after surgery (Floyd et al., 2014; Giannobile et al., 2015; Silva, Cortez, Moreira, & Mazzonetto, 2006).

Tissue engineering has been implemented as a method of avoiding some of the limitations of bone grafting. Guided tissue regeneration is on such example and involves the use of a barrier device or membrane in conjunction with grafting. The membrane is placed in an attempt to, “provide conditions that facilitate ingrowth of cells from the ligament while excluding those derived from epithelium and gingival connective tissue” as seen in **Figure 1** (Floyd et al., 2014). This idea of controlling which cells are allowed into the area of a defect has been carried further into the development of scaffolds.

Scaffolds are three-dimensional template structures that physically support and facilitate regeneration of periodontal tissue (Rios, Lin, Oh, Park, & Giannobile, 2011). They are one of the three key elements to tissue regeneration; the other two elements being cells and signaling molecules (Hasegawa et al., 2006). The reals strength of scaffolds is their ability to not only act as a physical barrier, but also as a delivery method for cells and signaling molecules. Recent research has led to the development of scaffolds that contain necessary cells and growth factors layered inside in the correct orientation to facilitate regeneration of tissues (Liu et al., 2019).

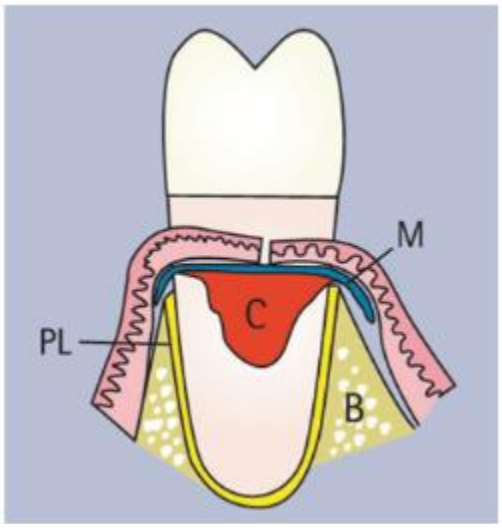

Figure 1 - Infrabony Defect and Exclusionary Membrane

Placement of an exclusionary membrane (M) allows Periodontal Ligament (PL) and Bone (B) growth into the defect. *Republished with permission from "Clinical guide to periodontology: Reconstructive periodontal treatment," by Floyd, P. D., Ide, M., & Palmer, R. M., 2014. British Dental Journal, 216(9), 511-518. permission conveyed through Copyright Clearance Center, Inc.*

As the technology and research into tissue engineering continues to progress, more and more serious lesions will be able to undergo regeneration. Laugish et al. recently examined the development of regeneration of Class II furcations over the last decade. They found that human histologic evidence shows periodontal regeneration in Class II furcations, but that there is little to no evidence of regeneration in Class III lesions (Laugisch et al., 2019). Though treatment has progressed drastically in the last decade, areas still remain where more development is not only possible but needed. Rios et al. identified the following areas as needing further study and development: finding new cell sources and clinically

relevant cell numbers, the ability to integrate added cells into existing tissue matrices, and expanding the biomaterials available to be used as tissue equivalents (Rios et al., 2011).

MURINE MODEL FOR PERIODONTITIS

Animal models are frequently used for research in situations when ethical considerations limit the use of human subjects. These animal models can be especially effective in studying underlying mechanisms of disease, in proving cause and effect relationships, and in testing the potential of new therapeutics (Graves, Dana T., Fine, Teng, Van Dyke, & Hajishengallis, 2008). Because no animal will perfectly replicate a human model, different animal models are used based on their advantages and disadvantages for each situation. Mice are a particularly helpful model due to their availability, the large number of strains with targeted genetic deletions (Graves, Dana T. et al., 2008). Mice are particularly suited for periodontal models as they share similarities to humans in anatomic, bacterial, and pathogenic periodontal characteristics (Saadi-Thiers et al., 2013). The ligature-induced periodontitis model is ideal for the study of periodontal disease in mice because it allows for the disease to be initiated and terminated at a known time (Abe & Hajishengallis, 2013). Furthermore, the resultant bacterial plaque on the ligature can be cultured and analyzed. One concern that has been expressed with this model is the possibility that the apparent alveolar bone loss is from trauma and not a result of the bacterial plaque on the ligature as is desired. It has been shown in recent studies that the bone loss is, in fact, a result of bacterial accumulation on the suture, and not the trauma of suture placement (Marchesan et al., 2018).

An additional point that must be taken into consideration, especially in murine models, is that responses can vary significantly in animals of different ages, sexes, and strains. The C57BL/67 strain of mice has been shown to be susceptible to Porphyromonas gingivalis lipopolysaccharide (LPS) induced bone loss and is ideal for use in studies concerning periodontal disease (Hiyari et al., 2015). Saadi-Thiers et al. demonstrated that the physiologic response of these mice to LPS induced bone loss is similar to the response of human patients with periodontal disease (Saadi-Thiers et al., 2013). They documented an increase in expression of matrix metalloproteinase-9 (MMP-9) and cathepsin B (CATB) when using ligature models. An increase in interleukin-6 (IL-6), IL-1β, and tumor necrosis factor-α (TNF-α) were also demonstrated. Though female mice are shown to be more susceptible to ligature-induced bone loss, male mice aged 6-12 weeks are typically used to avoid any alteration to results introduced by the presence of estrogen (Li & Amar, 2007; Saadi-Thiers et al., 2013).

CLINICAL APPLICATIONS OF VIBRATION THERAPY

Vibration therapy has been studied for years as a potential non-pharmacological therapy for a wide variety of conditions, especially regarding treatment of the musculoskeletal system. Some areas for which it may be useful are skeletal wound healing, dental extractions, periodontal disease, bone graft integration, recovery after stroke, osteoporosis and osteopenia, and healing of diabetic wounds (Alikhani, M. et al., 2016; Edwards & Reilly, 2015; Rubin, Judex, & Qin, 2006; Thompson, Yen, & Rubin, 2014; Weinheimer-Haus, Judex, Ennis, & Koh, 2014). Though it could potentially be utilized in so many different situations, one of the major limitations of vibration therapy is the identification of the ideal frequency and standardizing it across experimental

models (Edwards & Reilly, 2015). It may be surprising that the musculoskeletal system responds to specific frequencies, but as Rubin, Judex and Qin explain, other systems such as sight, touch, and hearing all function in the same way (Rubin et al., 2006). The current school of thought is to replicate the persistent high-frequency, low-magnitude signals that the musculoskeletal system experiences throughout a normal day. This falls somewhere in around the range of the range of 15-90 Hz with an acceleration less than that due to gravity (1.0 g), and a magnitude of less than 1 mm (Edwards & Reilly, 2015; Thompson et al., 2014; Zhang et al., 2015).

In the absence of inflammation, vibration therapy has been shown to have an anabolic, or bone building, effect on the skeleton (Alikhani, M. et al., 2016; Alikhani, Mani et al., 2018). As can be expected, studies have shown that this is accomplished through the activation of osteocytes leading to up-regulation of osteoblasts and down-regulation of osteoclasts (Alikhani, M. et al., 2016; Edwards & Reilly, 2015; Moustafa et al., 2012; Zhou et al., 2015). On a molecular level, the effects of vibration therapy signaled molecules in the mechanotransduction pathways including a decrease in RANKL and RANK mRNA and an increase in growth factors and Prostaglandin E2 (Alikhani, M. et al., 2016; Benjakul, Leethanakul, & Jitpukdeebodintra, 2019; Weinheimer-Haus et al., 2014).This anabolic effect was not limited to osteocytes, but also involved stem cells such as mesenchymal stem cells (MSCs) and periodontal ligament stem cells (PDLSCs) (Edwards & Reilly, 2015; Zhang et al., 2015). Not only were stem cells able to be induced to commit to an osteoblast lineage through vibration therapy, but there was an inversely coupled relationship between pre-osteoblasts and pre-adipocytes (Luu et al., 2009).

VIBRATION THERAPY WITHIN DENTISTRY AND ORTHODONTICS

One of the most popular current applications of high-frequency vibration in the craniofacial region is use in conjunction with orthodontic therapy. There are multiple commercially available products that promise to reduce the treatment time and discomfort of orthodontic treatment; some promise as much as a 64% reduction in length of treatment (Propel Orthodontics, 2020). The reason behind this trend as well as the treatment claims is that some studies have demonstrated an upregulation in cytokines and other molecules that contribute to the inflammation-dependent catabolic cascade that enables orthodontic tooth movement (Alikhani, Mani et al., 2018; Benjakul et al., 2019; Phusuntornsakul, Jitpukdeebodintra, Pavasant, & Leethanakul, 2018). However, other studies have reported contradictory findings and claim that, as it does in the musculoskeletal system as a whole, high-frequency vibration increased bone density and down-regulates resorption in alveolar bone (Benjakul et al., 2019; Kalajzic et al., 2013; Sakamoto et al., 2019). As mentioned previously in this review, one of the challenges of researching vibration and its effects on alveolar bone and tooth movement is the lack of standardization of vibration methods such as force magnitude, frequency, exposure, duration, and timing (Alikhani, Mani et al., 2018; Sakamoto et al., 2019). This variance in methods could explain the differing reports. Many of the studies use lower frequency vibrations which are known to have less of an effect on tooth movement than higher frequencies (Alikhani, Mani et al., 2018; Sakamoto et al., 2019; Yadav et al., 2015). Ideally, the different data will aid in the isolation of ideal frequencies for tooth movement as well as bone anabolism.

STUDY AIMS

To establish a mouse periodontitis model and use this model to explore the optimal time window to investigate the effects of mechanical vibration on alveolar bone following experimental periodontitis in mice.

CHAPTER III – MATERIALS AND METHODS

ANIMAL MODEL AND STUDY PROTOCOL

This project is an extension of the work done by Dr. Andrei Taut for his thesis at Marquette University using the same murine samples and measurement protocols, but with additional data, new measurements, and more comprehensive comparisons. As he designed and carried out the animal protocols, much of this section is taken from his thesis.

Adult male C57BL/67 (n = 95, average weight 21–26 g, 11 weeks old) were housed and treated according to a protocol conforming to ARRIVE (Animal Research Reporting of the In Vivo Experiments) guidelines and approved by the Marquette University Institutional Animal Care and Use Committee (IACUC). The timeline of the experiment is detailed in **Figure 2**. Two weeks following arrival at Marquette University, the animals were assigned into four groups: 1) Healthy Group that served as the control with no intervention, 2) Healthy + Vib group that received HFMV for 7 and 21 days, respectively (frequency = 60 Hz, acceleration = 0.3 g where 'g' represents the acceleration of gravity (1 g = 9.81 m/s^2)) for 5 minutes per day, 3) Perio Group which had sterilized silk sutures/ligatures placed according to protocol below and received no other intervention; and 4) Perio + Vib Group that received silk ligatures and HFMV for 7 and 21 days, respectively (frequency = 60 Hz, acceleration = 0.3 g where 'g' represents the acceleration of gravity (1 *g* = 9.81 m/s^2)) for 5 minutes per day. The 5-minute daily

duration of HFMV was chosen to remain consistent with previous internal and external experimental designs (Alikhani et al., 2019; Alikhani et al., 2018).

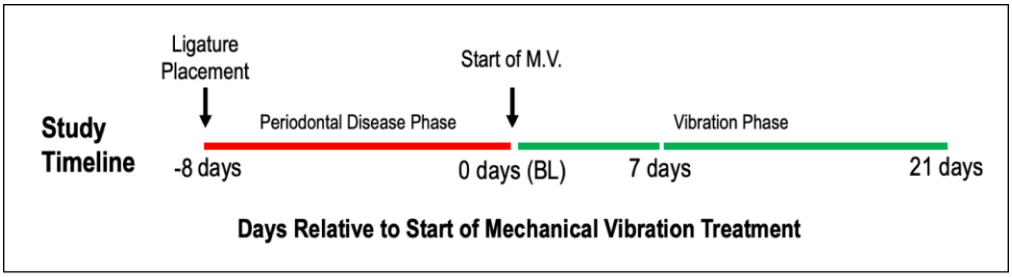

Figure 2 - Study Timeline
Study timeline began with 8 days of experimental periodontitis, followed by ligature removal (Day 0) and 7 and 21 days of HFMV treatment (HFMV treatment initiated 24 hours following removal of ligatures).

In accordance with the Recommended Best Practices for Mouse Anesthesia designed by the Marquette University's Office of Research and Compliance (https://www.marquette.edu/orc/animal-care-use/documents/AnestheticsandAnalgesicsRodent2017.pdf), the animals were anesthetized using isoflurane inhalation (Charles River Laboratories International, Inc.). Using the Simplified Ligature Model Materials – custom 3-D printed mouse dental bed and 3-D printed U-tipped ligature holder (Marchesan Lab, University of North Carolina Adams School of Dentistry, Chapel Hill, NC, USA) seen in **Figure 3**, silk sutures (5/0) were placed unilaterally into the interproximal gingival sulci of the right maxillary 1st and 2nd molar teeth according to previously described protocol (Marchesan et al., 2018) to induce experimental periodontitis. Sutures were checked every other day to ensure their presence and were replaced as necessary. Experimental periodontitis was induced for a period of 8 days. Intact controls (Healthy) were not ligated and served as controls. Ligatures were removed at the end of the experimental periodontitis phase before HFMV

treatment. HFMV treatment was initiated 24 hours following ligature removal to allow for the inflammatory response to subside.

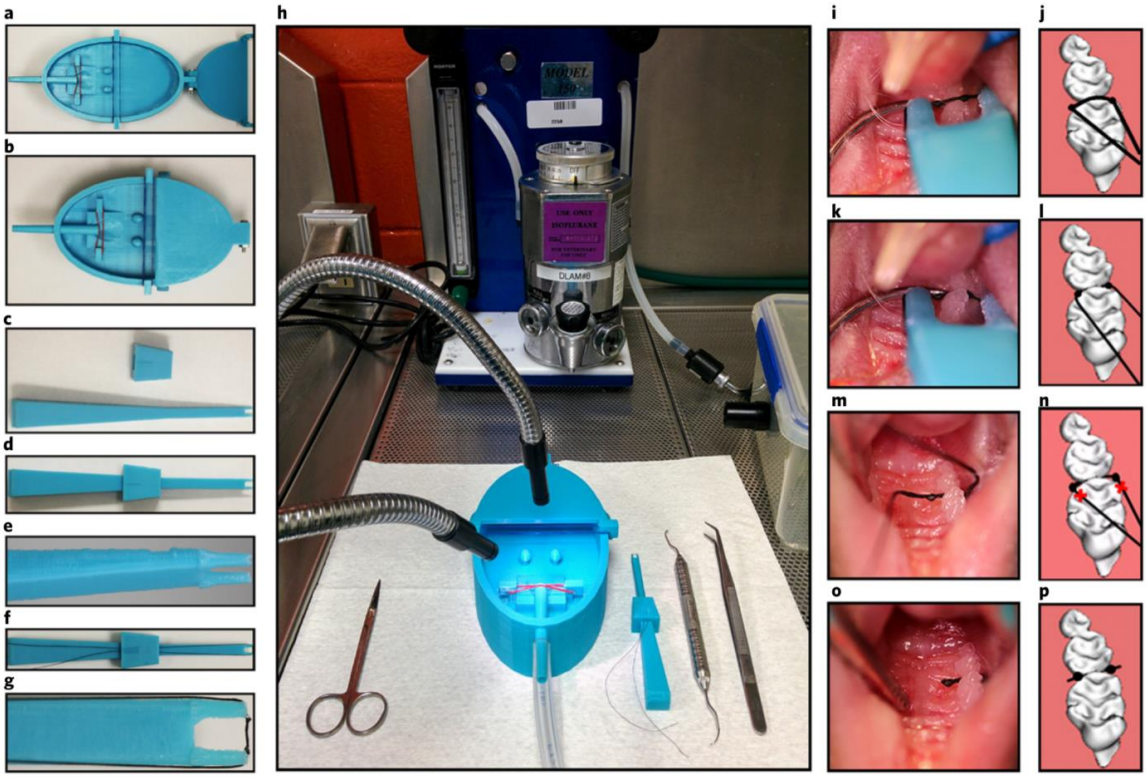

Figure 3 - Tools and technical procedures required to set up the simplified ligature model in mice

The tools required. **a**, Mouse dental bed. **b** represents high magnification of **a. c**, U-tipped ligature holder (U-shaped for holding silk). **d**, Assembled U-tipped ligature holder. e represents high magnification of U-tipped ligature holder. **f**, The U-tipped holder with 5-0 silk suture. g, High-magnification view of the U-tipped holder, showing two knots in the inside of the forceps tips (~2.5-mm distance between knots). **h**, Experimental setup immediately before anesthetizing the mouse with isoflurane. **i–p**, The stages required to insert the ligature are shown as photos (**i**,**k**,**m**,**o**) and diagrammatically (**j,l,n,p**). **i,j**, The left hand is used to hold the dental explorer while the tip of the dental explorer and the 2.5-mm silk between the knots are carefully located in the gap between the 1st and 2nd molars, using the U-tipped ligature holder held in the right hand. **k,l**, The suture is then pushed through the interdentium between the 1st and 2nd molars. **m,n**, The silk is cut, and the U-tipped forceps are removed. **o,p**, Finally, the silk is trimmed at the end of the knot. Appropriate institutional regulatory board permission was obtained to carry out the experimental procedure on the mouse shown here." *Republished with permission of Springer Nature from "An Experimental Murine Model to Study Periodontitis," by Marchesan J.T., et al, 2018, October, Nature Protocols, 13(10):2247-2267; permission conveyed through Copyright Clearance Center, Inc.*

MECHANICAL VIBRATION APPLICATION AND FLUORESCENT BONE LABELING

In accordance with the Recommended Best Practices for Mouse Anesthesia designed by the Marquette University's Office of Research and Compliance (https://www.marquette.edu/orc/animal-care-use/documents/AnestheticsandAnalgesicsRodent2017.pdf), animals were anesthetized using isoflurane inhalation (Charles River Laboratories International, Inc.) and unilateral mechanical vibration was conducted through an electromechanical actuator held in place by a custom apparatus as demonstrated in the diagrammatic representation in **Figure 4**. LabView Custom software (National Instruments, Austin, TX) was designed to communicate with the electromechanical actuator to produce the specific vibration frequencies. Vibration was conducted at 0.3 g (acceleration), 20 micrometers of micro-vertical displacement, and 60 Hz frequency, for 5 min/day for 7 days and 21 days. The 7-day experimental period for early assessment of the effects of HFMV on inflammation, as well as on osteogenic and bone resorptive signaling cascades. Previous studies demonstrated a statistically significant increase of alveolar bone starting at 14 days after initiation of HFMV and up to 56 days of HFMV, thus the 21-day experimental period was selected as the practical way to assess the long term effects of HFMV on alveolar bone following experimental periodontitis (Alikhani et al., 2016). During the 21-day experimental period, mice were given two fluorescent markers — Calcein (50 mg/kg

body weight) and Alizarin Red (50 mg/kg body weight) – at days 7 and 14, by subcutaneous injection (total of 2 injections).

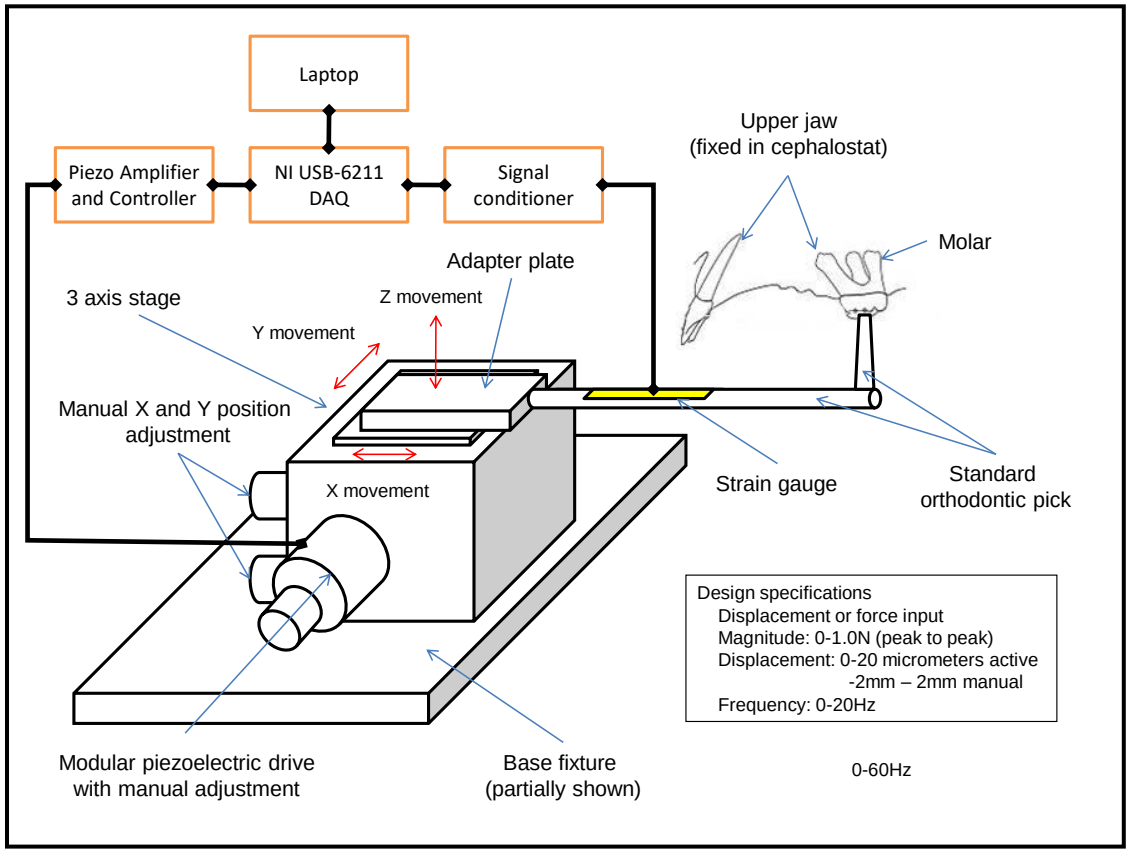

Figure 4 - Diagram of Mechanical Vibration Set-Up
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MICRO-COMPUTED TOMOGRAPHY SCANNING AND BONE ANALYSIS

Maxillary bone tissues – including the 1st, 2nd, and 3rd molars – were collected at the designated end points, placed in 10% neutral-buffered formalin for 2 days, and transferred to 70% ethanol (EtOH) for microCT scanning. Formalin-fixed maxillae were subjected to micro-computed tomography (CT) image analysis. The specimens were

scanned in all three spatial planes at a resolution of 8 x 8 x 8 μm (μCT40, Scanco Medical, Brüttisellen, Switzerland) as previously described (Park et al., 2007). Peak voltage was set to 55 kVp. To assess alveolar bone loss, the distance between the cementoenamel junction (CEJ) and alveolar bone crest (ABC) was measured at two sites for the 1st molars (disto-palatal and disto-buccal) and two sites for the 2nd molars (mesio-palatal and mesio-buccal) in three-dimensional images viewed from the buccal and palatal sides as described (Park et al., 2007) and detailed in **Figure 5A**. Using MicroView 2.5.0-rc25 software (Parallax Innovations Inc., Ilderton, ON, Canada), each reconstructed image was rotated into a standardized orientation, and a region of interest (ROI) for each specimen was created as shown in **Figure 5B**. Briefly, for volumetric analysis of the maxillary tooth-supporting alveolar bone, the inter-radicular alveolar ridge crests, inter-radicular surfaces of the roots of the maxillary 1st and 2nd molars, cemento-enamel junction, and root apex of the mesio-buccal root of the 1st maxillary molar and disto-buccal root of the 2nd maxillary molar were used as landmarks for quantifying alveolar bone loss and regeneration within a reproducible region **Figure 5B**. Using the average Grayscale threshold value for all of the samples, the alveolar bone interproximally between the 1st and 2nd maxillary molars, and the inter-radicular bone area of the maxillary 1st and 2nd molars, including bone volume fraction (BVF) and tissue mineral density in mg/cc (TMD) were quantified. TMD was used because it describes the density of the bone itself and does not include the surrounding soft tissue.

Intra-examiner reliability was a concern in this study given that measurements were performed by one examiner. To evaluate reliability, random samples were selected

and then remeasured at three different time points over a period of five months after completing initial measurements.

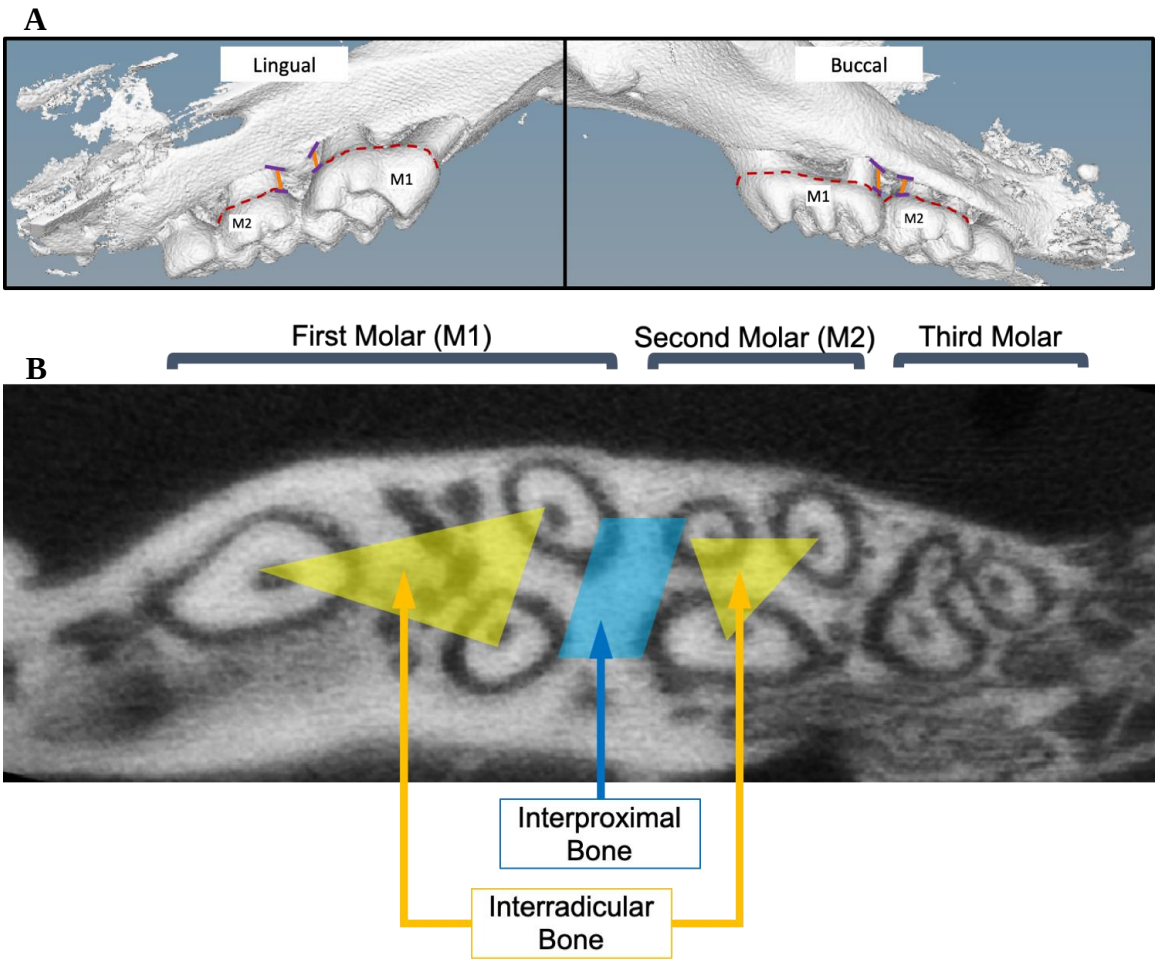

Figure 5 - Linear and Three-Dimensional Regions of Interest
A. Linear alveolar bone loss (ABC-CEJ), or the linear distance (orange line) between the cementoenamel junction (CEJ; maroon-dashed line) and alveolar bone crest (purple line), was measured along two roots for M1 and two roots of M2. B. Anatomical landmarks of M1 and M2 were used to create a three-dimensional ROI encompassing the inter-radicular bone (yellow triangles) and interproximal bone (blue parallelogram).

STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism software. Data were pooled by experimental group, and the mean, standard deviation, and standard error were calculated. Intraclass Correlation Coefficient (ICC) was calculated using the two-way mixed effects model.

Analysis of variance (ANOVA) was performed with Bonferroni post hoc tests to measure statistically significant differences between groups for volumetric and linear bone levels. Mean and standard error of were plotted in bar graphs and line charts were utilized for linear measurements. For this research, a *P* value less than 0.05 was considered as statistically significant.

CHAPTER IV – RESULTS

Intraclass Correlation Coefficient was calculated to be 0.9996, indicating an excellent reliability.

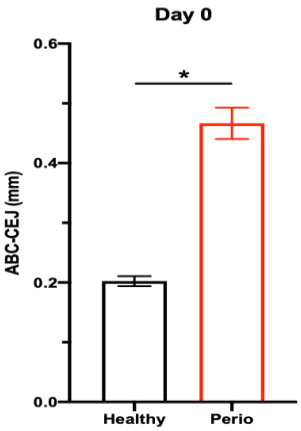

Figure 6 - Linear Bone Height (ABC-CEJ) at Day 0
Day 0 ABC-CEJ values for all four roots were pooled into one graph due to the limited variability. Linear bone height (ABC-CEJ) significantly reduced after 8 days of ligature placement at all four sites. * indicates statistical significance of $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc tests.

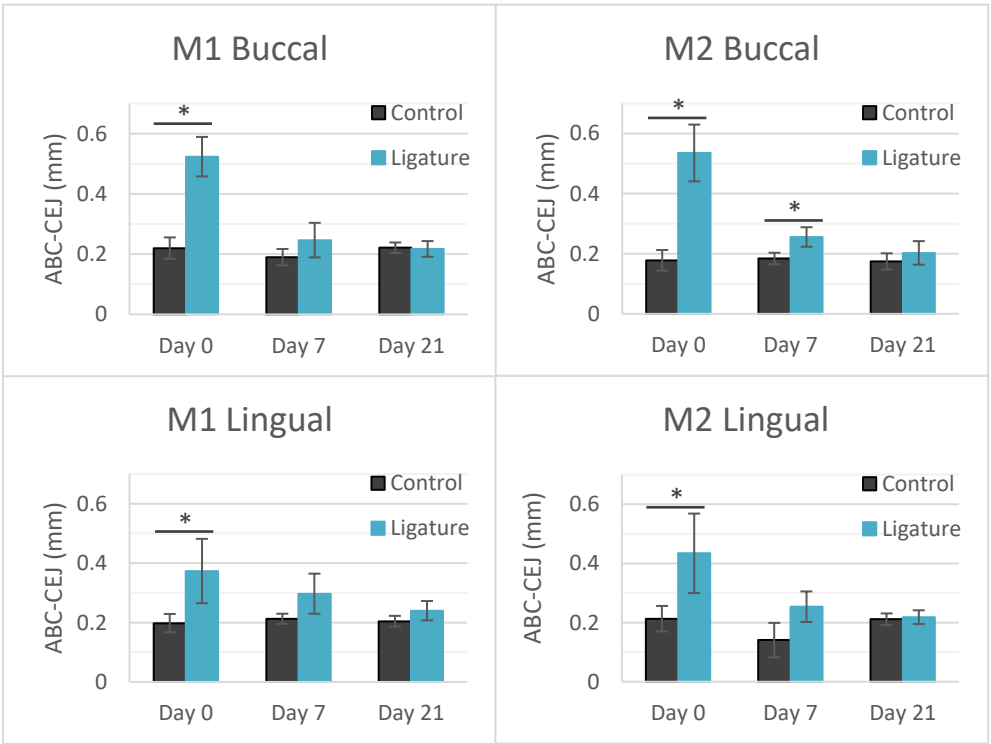

Figure 7 - Linear Bone Loss (ABC-CEJ) at Day 0, 7, and 21
Linear bone height (ABC-CEJ) significantly reduced after 8 days of ligature placement at all four sites. Bone height is recovering at 7 days and at healthy levels after 21 days. * indicates statistical significance of $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc tests.

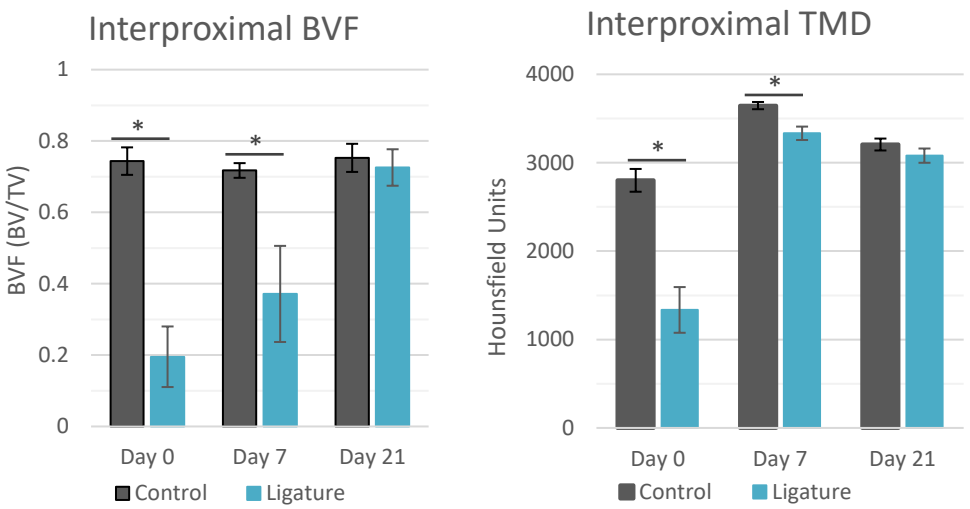

Figure 8 - Interproximal bone volume (BVF) and density (TMD)
Alveolar bone volume (BVF) and tissue mineral density (TMD) significantly reduced interproximally at site of ligature placement between M1 and M2. * indicates statistical significance of $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc tests.

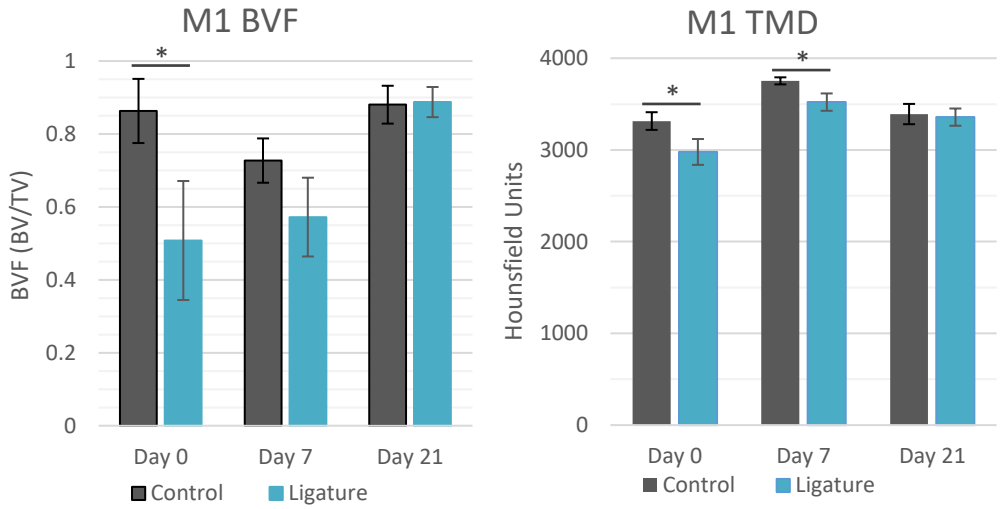

Figure 9 - First molar inter-radicular bone volume (BVF) and density (TMD)
Alveolar bone volume (BVF) and density (BMD) significantly reduced at adjacent site – inter-radicular region of M1. * indicates statistical significance of $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc tests.

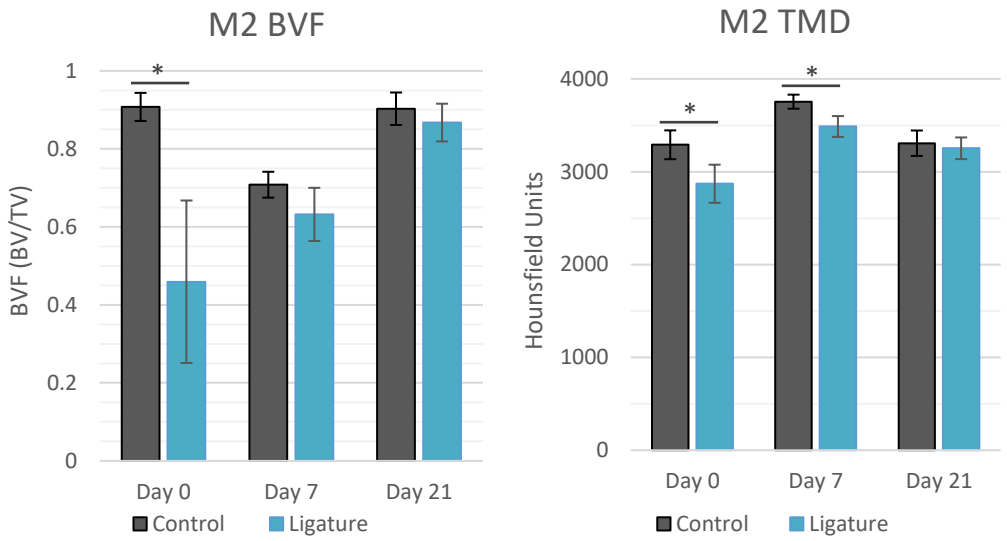

Figure 10 - Second molar inter-radicular bone volume (BVF) and density (TMD)
Alveolar bone volume (BVF) and density (BMD) significantly reduced at adjacent site – inter-radicular region of M2. * indicates statistical significance of $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc tests.

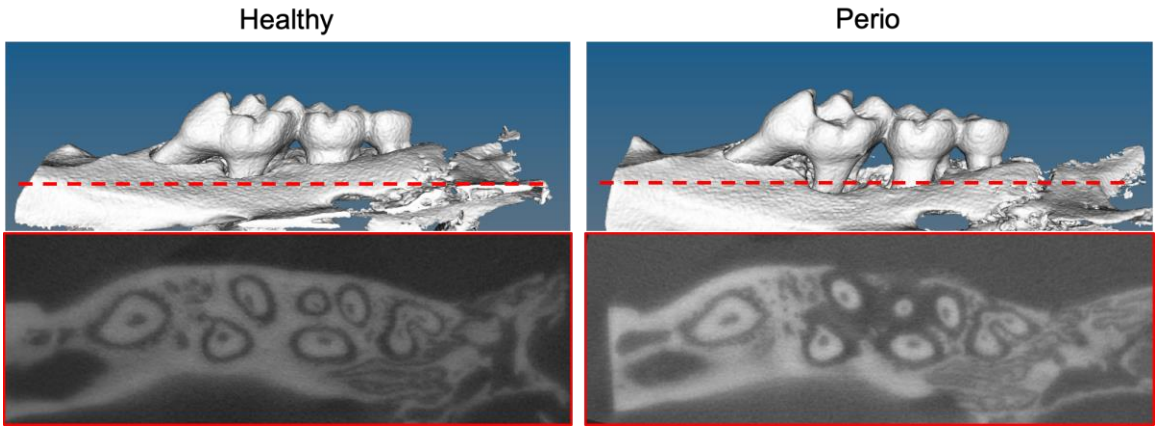

Figure 11 - Alveolar Bone Loss Following Experimental Periodontitis
Representative micro-CT images of maxillary alveolar bone surrounding the 1st (M1) and 2nd (M2) molars at Day 0 for healthy and experimental periodontitis groups. Representative coronal slices (2D) as well as 3D images of maxillary specimens showcase the visual differences between the amount of bone resorption following 8 days of ligature-induced periodontitis.

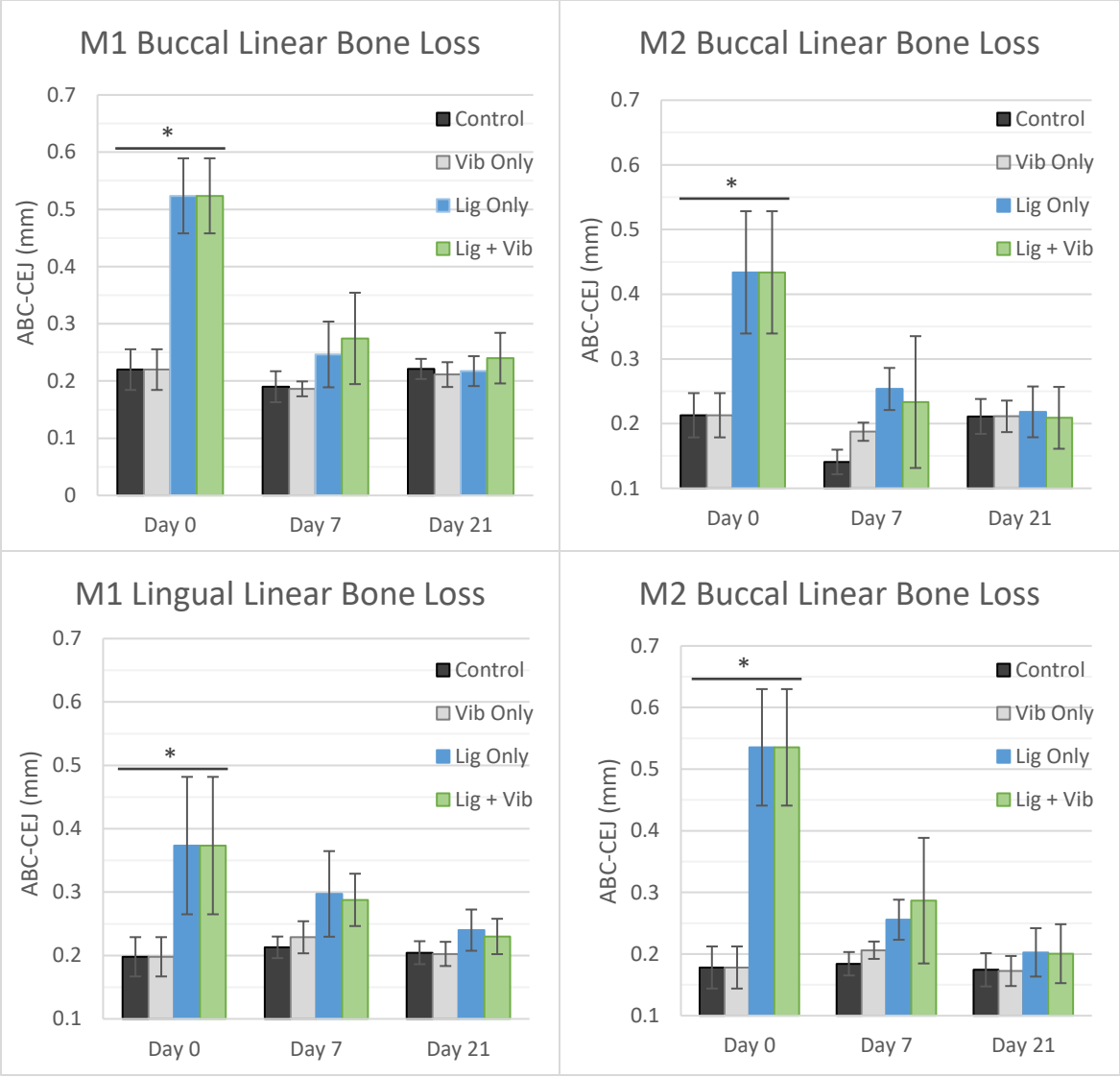

Figure 12 - Linear Bone Loss (ABC-CEJ) at Day 0, 7, and 21

Non-significant decrease in bone loss after application of HFMV for 21 days when comparing vibration only with control as well as ligature + vibration with ligature only. Because vibration did not start until Day 0, values for vibration only and control were assumed to be the same at Day 0. The same is true for ligature only and ligature + vibration. * indicates statistical significance of $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc tests.

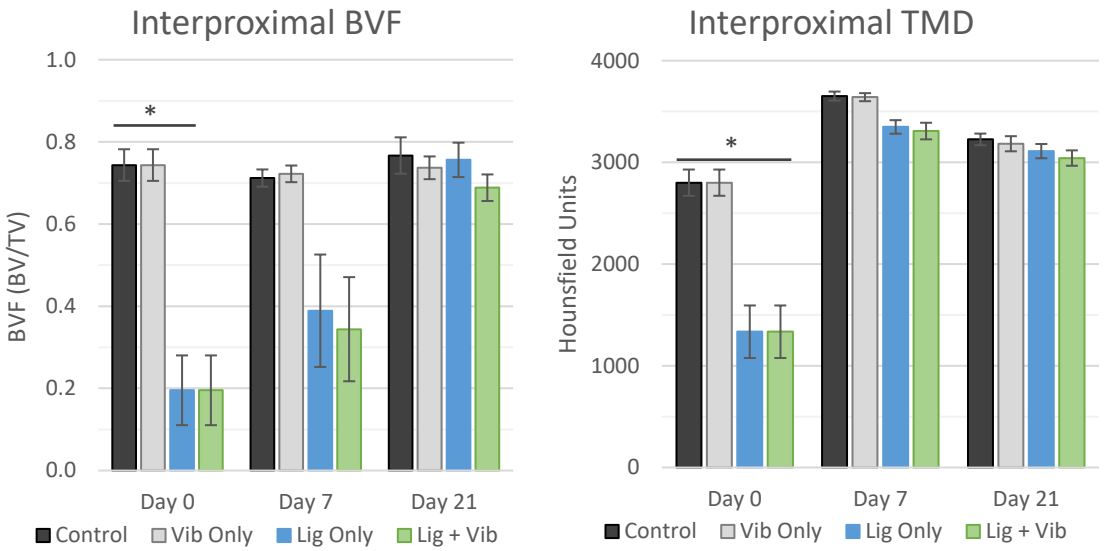

Figure 13 – Interproximal bone volume (BVF) and density (TMD)

Non-significant increase in bone volume and density interproximally at site of ligature placement between M1 and M2 after application of HFMV for 7 and 21 days when comparing vibration only with control as well as ligature + vibration with ligature only. Because vibration did not start until Day 0, values for vibration only and control were assumed to be the same at Day 0. The same is true for ligature only and ligature + vibration. * indicates statistical significance of $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc tests.

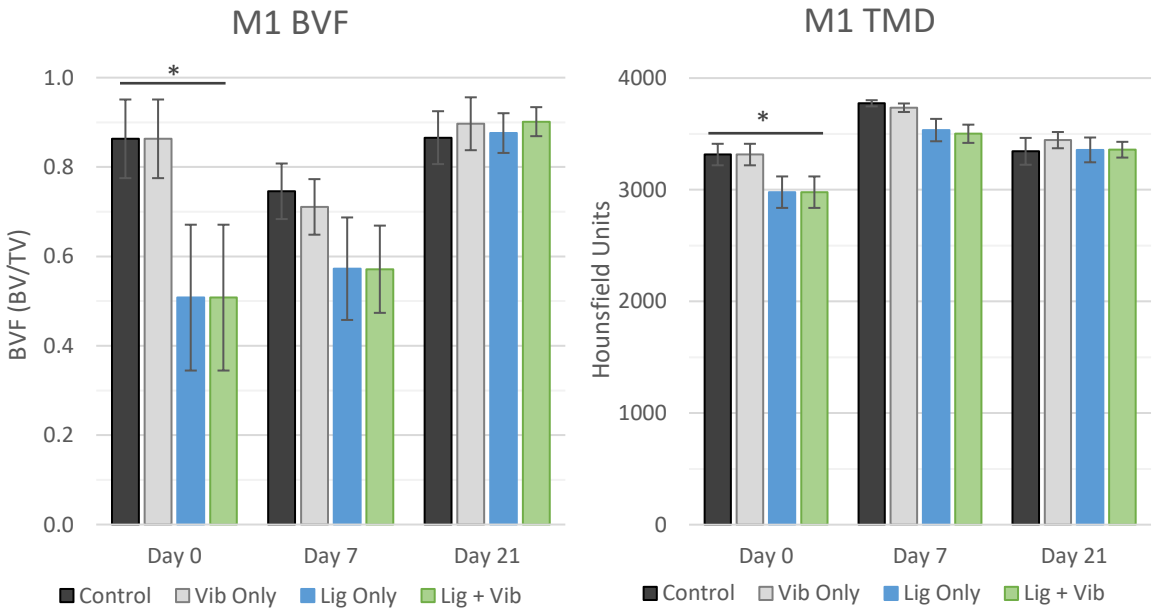

Figure 14 - First molar inter-radicular bone volume (BVF) and density (TMD)

Non-significant increase in bone volume and density at adjacent site – inter-radicular region of M1 after application of HFMV for 7 and 21 days when comparing vibration only with control as well as ligature + vibration with ligature only. Because vibration did not start until Day 0, values for vibration only and control were assumed to be the same at Day 0. The same is true for ligature only and ligature + vibration. * indicates statistical significance of $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc tests.

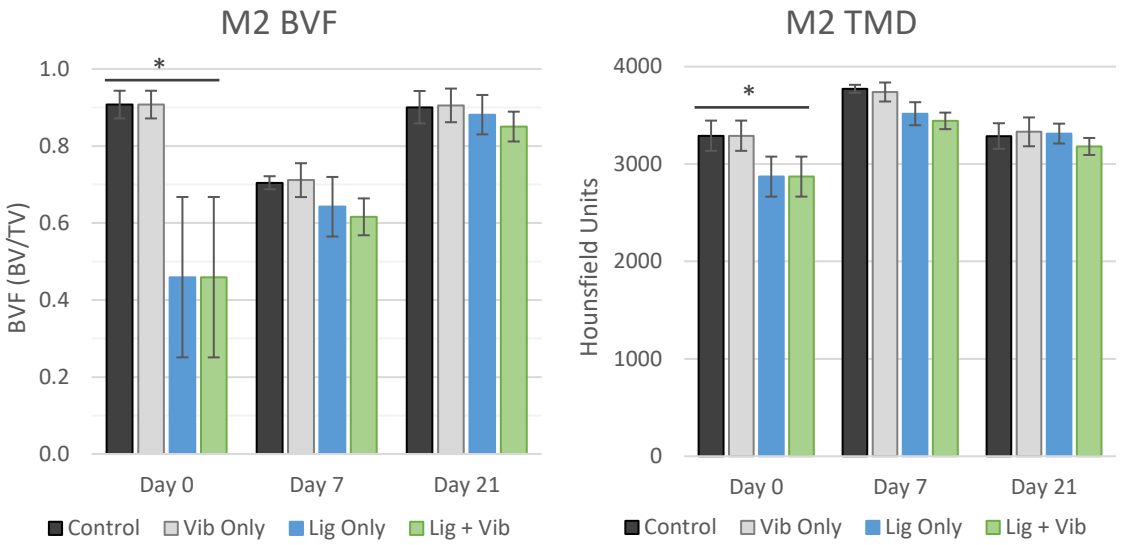

Figure 15 – Second molar inter-radicular bone volume (BVF) and density (TMD)

Non-significant increase in bone volume and density at adjacent site – inter-radicular region of M2 after application of HFMV for 7 and 21 days when comparing vibration only with control as well as ligature + vibration with ligature only. Because vibration did not start until Day 0, values for vibration only and control were assumed to be the same at Day 0. The same is true for ligature only and ligature + vibration. * indicates statistical significance of $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc tests.

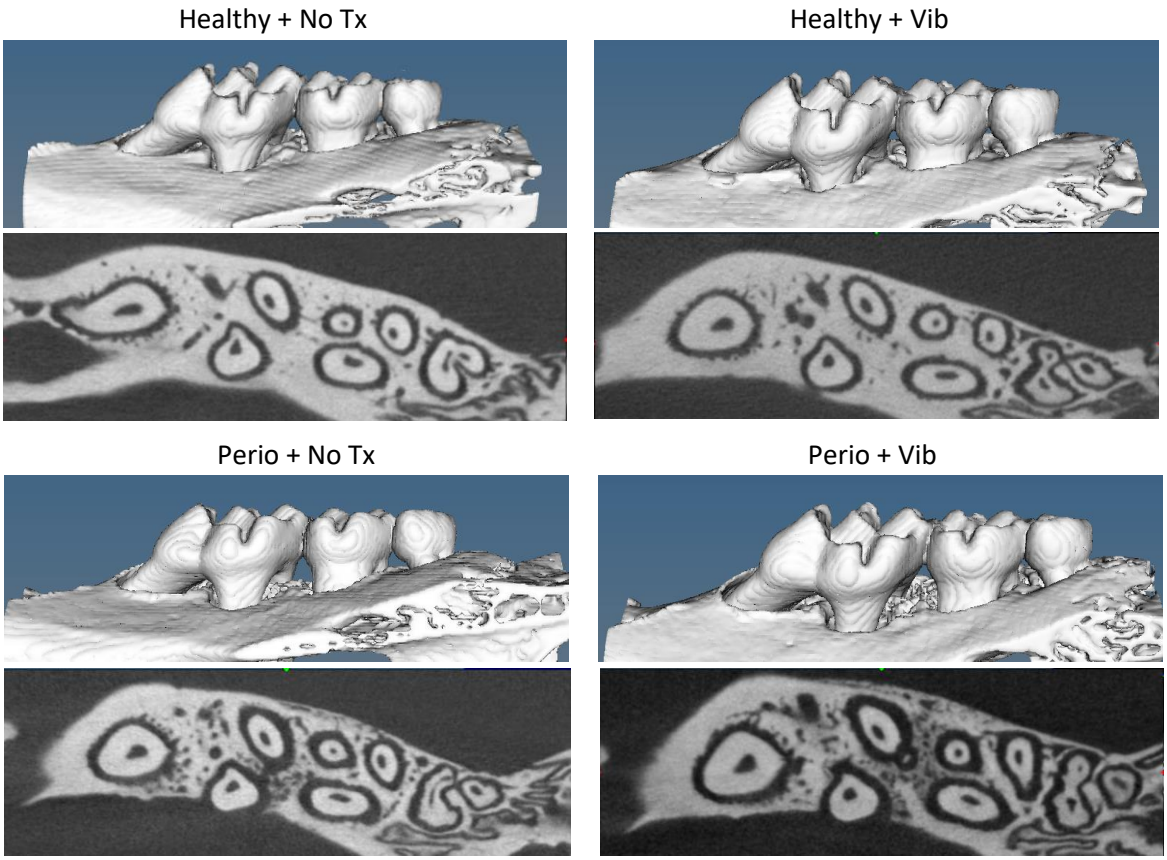

Figure 16 - Alveolar Bone Following 7 Days of HFMV Treatment

Representative micro-CT images of maxillary alveolar bone surrounding the 1st (M1) and 2nd (M2) molars at Day 7 for healthy, experimental periodontitis, and HFMV-treated groups. Representative coronal slices (2D) as well as 3D images of maxillary specimens showcase the relatively minor differences between the tooth supporting alveolar bone in the control and treatment groups.

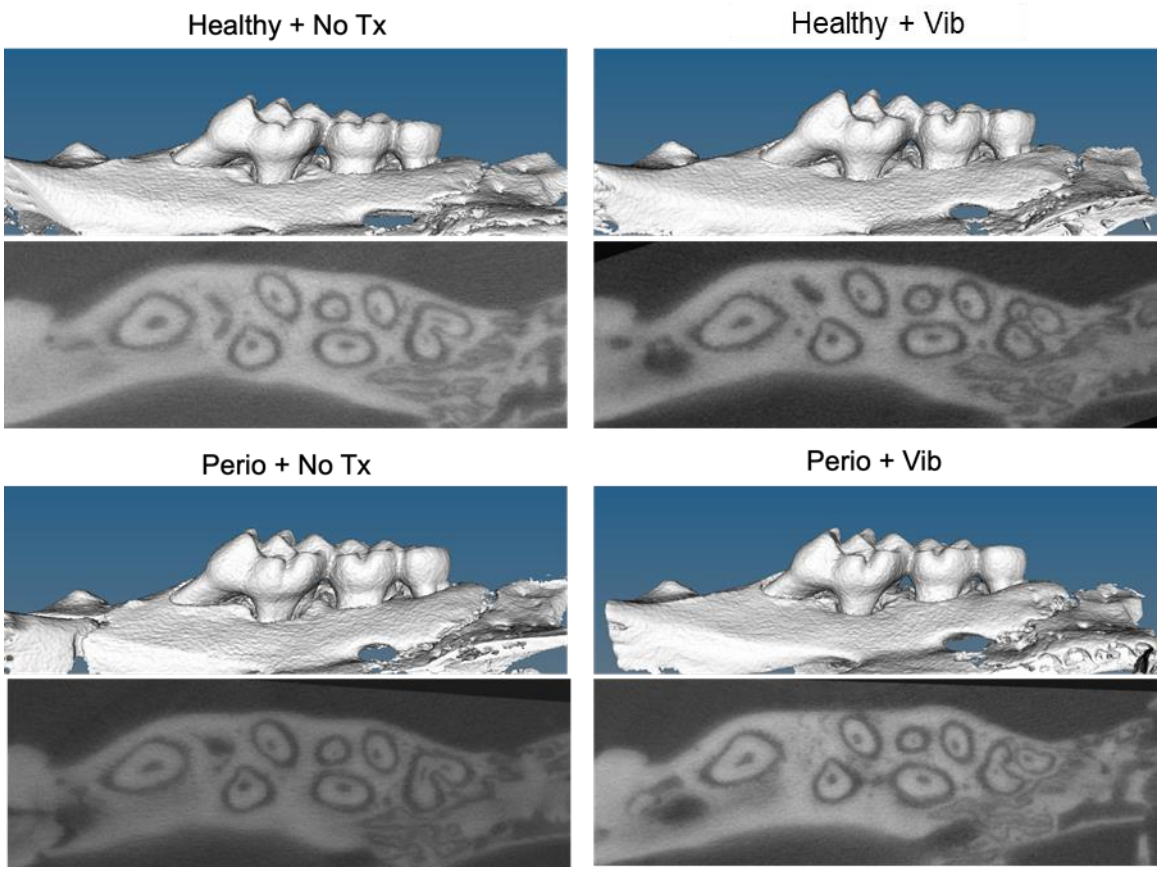

Figure 17 - Alveolar Bone Following 21 Days of HFMV Treatment
Representative micro-CT images of maxillary alveolar bone surrounding the 1st (M1) and 2nd (M2) molars at Day 21 for healthy, experimental periodontitis, and HFMV-treated groups. Representative coronal slices (2D) as well as 3D images of maxillary specimens showcase the relatively minor differences between the tooth supporting alveolar bone in the control and treatment groups.

CHAPTER V – DISCUSSION

The purpose of this study was to establish a mouse periodontitis model and use this model to explore the optimal time window of the periodontal healing after periodontitis to investigate the effects of mechanical vibration on alveolar bone following experimental periodontitis in mice. The murine periodontitis model is an ideal method for the study of periodontal disease. Benefits include wide availability of animals, the presence of strains with targeted genetic deletions, and the similarities to humans in anatomic, bacterial, and pathogenic periodontal characteristics (Graves, Dana T. et al., 2008; Saadi-Thiers et al., 2013). Of the available murine periodontitis models, the ligature-induced model is ideal for the study of periodontal disease because it enables researchers to initiate and terminate periodontal disease at a known time (Abe & Hajishengallis, 2013). As mentioned previously, the animal models for this study were designed and carried out by Dr. Andrei Taut for his master's thesis. Similarities in many of the discussions are sure to be noted; however, this study accounts for additional data from the day 7 that merited further investigation to provide further insight into periodontal healing and the effects of mechanical vibration.

One notable difference between Dr. Taut's thesis and this study is the use of tissue mineral density (TMD) as opposed to bone mineral density (BMD). Bone mineral density is a measure of the amount of bone in relation to the surrounding soft tissue, it does not provide information about the density of the bone that is present. In contrast, tissue mineral density is a measurement of the density of the bone that is present, it gives no information about the surrounding soft tissue. Because of the inclusion of bone volume fraction which measures voxels of bone compared to total voxels, TMD was

selected for this study to provide information about the density of the bone (Bouxsein et al., 2010).

As established in previous studies, the placement of ligatures induced statistically significant bone loss in the area of ligature placement (interproximal between maxillary 1st and 2nd molars) and extended to the interradicular areas of adjacent molars (Abe & Hajishengallis, 2013; Marchesan et al., 2018). Bone loss was demonstrated after 8 days in all three of our measurements: alveolar bone height, bone volume, and bone density. The degree of bone loss demonstrated, combined with the size of the affected area, indicate that, as had been shown in previous studies, the ligature model causes bone loss through bacterial accumulation at the site of ligature placement (Abe & Hajishengallis, 2013; Graves, Dana T. et al., 2008; Marchesan et al., 2018). The data from day 7 showed that, in the case of BVF and linear measurements, healing was progressing, but not complete. TMD was different as all three locations showed that the bone density at 7 days was as high as the density at 21 days. This pattern can be noted in both the ligature only and the ligature + vibration groups, so it cannot be attributed to vibration. One possible explanation is that while the alveolar bone height and bone volume need more than 7 days to fully recover, the bone density may recover more rapidly. This can be interpreted to mean that the quality of bone rapidly improves after periodontitis, but the volume and bone height take more time to recover.

The application of localized high-frequency, low magnitude mechanical vibration did not have a significant effect on healing in any of the areas or values measured. However, as was noted by Taut, there is an interesting trend in the ligature + vibration group when compared to the ligature only group. The application of HFMV (60 Hz, 0.3

g, 5 min/day) after suture removal caused an increase in bone volume and bone density after 21 days. Interestingly, this effect was only seen at the site of application of vibration, the maxillary 1st molars. The opposite effect was seen at the other, more distant sites. The 7-day data show the same trend. Other studies have also shown HFMV has an anabolic effect on alveolar bone. Using the same protocol as in this study (60 Hz, 0.3 g, 5 min/day), Alikhani et al. found a 20% increase in bone volume after 28 days of vibration (Alikhani, M. et al., 2012). Despite having found a similar trend, this study cannot be directly compared to that of Alikhani et al. as they used Sprague-Dawley rats instead of mice.

The observed catabolic affect at the interproximal and 2nd molar sites is contrary to what other studies have shown. In the same study performed by Alikhani et al, the greatest anabolic effect was found at the site of HFMV application, but an increase in bone volume at both the interproximal and 2nd molar regions was noted (Alikhani, M. et al., 2012). The reason for different results in our study is not clear. Two possibilities are the use of different species and the lack of strain measurements in this study. The frequency, acceleration, and time were the same, but because strain is affected by alveolar bone and the PDL, it can vary despite using the same methods.

In his analysis of day 0 and day 21 data, Dr. Taut introduced a theory for the contrasting observed effects HFMV had on the various locations we studied and it merits being repeated here. Marchesan et al. demonstrated in their murine model of periodontitis that the inflammatory response peaks after 9 days (2018). In our study, HFMV was initiated after ligature removal at day 8. It is possible that, despite ligature removal, the inflammatory response was still significant enough to cause HFMV to have a catabolic

effect on alveolar bone. This theory could further be supported if strain measurements were taken as a part of our study. This limitation will be further explored below.

LIMITATIONS OF THE STUDY

As with any study, limitations for this study are more apparent in hindsight. There are multiple limitations that could have affected our study. First is the lack of strain measurements as mentioned before. Despite calibration of the device for mechanical stimulation, its direct effect on alveolar bone is not known without measuring strain distribution which can vary depending on location and method of application as well as species and degree of inflammation. Strain measurements would allow for the optimization of the HFMV regimen. A second limitation in this study is the lack of an established pattern for study length. Our estimate that 21 days would be an appropriate amount of time for this study was not inaccurate; however, after gathering the data, it is apparent that full recovery in our murine model occurs somewhere between 7 and 21 days. This recovery is quick enough that the full effect of HFMV may not be able to be seen as Alikhani et al. found the most significant increase in bone volume after 28 days of HFMV in their rat model (2016). In future studies this limitation may be corrected by leaving ligatures in place for longer than 8 days to stimulate a more chronic inflammatory patter which may increase the healing period beyond 21 days.

CONCLUSION AND CLINICAL IMPLICATIONS

Mechanical vibration (60 Hz, 0.3 g, 5 min/day) slightly increases bone volume and density in the region of application directly to the 1st molar. This research indicates that improving bone quantity and quality following periodontitis is a possible application

of HFMV. Our data demonstrate a more rapid effect on density than volume which would benefit from further investigation. Another potential application based on the anabolic potential of HMFV would be as a supplemental treatment for periodontal therapies involving scaffolds, growth factors, and autologous cells.

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